Peptide pores in lipid bilayers : voltage facilitation pleads for a revised model

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We address the problem of antimicrobial peptides that create pores in lipid bilayers, focusing on voltage-temperature dependence of pore opening. Two novel experiments (voltage-clamp with alamethicin as an emblematic representative of these peptides and neutron reflectivity of lipid-monolayer at solid/water interface under electric field) serve to revise the only current theoretical model [2]. We introduce a general contribution of the electric field as being responsible for an unbalanced tension of the two bilayer leaflets and we claim that the main entropy cost of one pore opening is due to the corresponding "excluded-area" for lipid translation.

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Interaction of living cell membrane with adsorbed molecules and the way their uptake occurs are at the heart of many biological issues. Among these molecules, antimicrobial peptides [1] attract special attention as being the keystone of the innate immune system of multicellular organisms. Antimicrobial peptides basically cause lipid-bilayer permeation by producing pores. Their universal presence in animal and plant kingdoms, their non-specific broad-spectrum and their elementary structure let us expect their action also obeys to a widespread and universal physical mechanism that probably puts the membrane behavior in central position.

The most accepted physical model for peptide pores opening is based on a tension-driven mechanism that can be summarized as follows [2]. Prior to pore formation, these amphipathic peptides adsorb parallel onto the membrane and are supposed to increase an "internal tension" up to a given adsorption level, beyond which they relax this tension by penetrating into the membrane, then stabilize the edge of pores that spontaneously appear in bilayers [3]. Although this model is the best attempt to formalize a widespread outlook, it is still unsatisfactory as it ignores two points : 1) the role of temperature and entropy ; 2) the role of the transverse electrical field of the order of 25×10^6 V/m experienced by living cell membranes that is known to be strong enough in some cases [4] to induce peptide-poration.

In this letter, we report two novel experiments : 1) voltage-clamp study focusing on the temperature-voltage dependence of pore opening with alamethicin as an emblematic representative of antimicrobial peptides [5] ; 2) neutron reflectivity of lipid-monolayer adsorbed at solid/water interface under electrical field. Our results demonstrate that the membrane behavior is central in this problem and the key role of its entropy. We support the idea that voltage-induced and zero-voltage peptide-poration obey the same physics : the former guiding us to grasp the latter. In this way, we propose a simple model that takes over some fundamentals of the tension-driven mechanism but solves some difficulties. In particular, we clarify the above "internal tension" as being due to a bending energy rather than a proper membrane tension ; we introduce a general contribution of the electric field as being responsible for an unbalanced tension of the two bilaler leaflets ; and finally we show that for a held membrane the main entropy cost of one pore opening comes from the corresponding exclude-area for the translational entropy of lipid molecules. The impact of these two last statements could be quite general, not only for pore opening but also for the energetics of incorporation of membrane proteins in lipid bilayers.

Voltage-clamp : Pore opening into lipid-bilayer was detected by voltage-clamp that consist in measuring the ionic current as a small electric potential is applied across a free-standing planar membrane, which separates two compartments containing KCl-1M aqueous solutions. Sample preparation and experiment setup are described in ref. [6]. Here, alamethicin-poration was studied on DPPC-bilayer versus temperature above the melting point of the lipid. This was checked by measuring at 10 Hz the temperature dependence of the resistance and the capacitance of the bilayer. Both are constant above 299 K : (4.3 ± 0.1) GΩ and (93 ± 1) pF, respectively. The latter leads to a bilayer-thickness $z = 50$ Å, assuming the relative permittivity of the membrane equal to 3 [7].

In our study, alamethicin was added only to one side of the membrane (*cis*-side) at a molecular ratio lipid/peptide of the order of 100 (estimated from the area to volume ratio of the device, the membrane area and the amount of peptides). At a given temperature T, the current intensity I was recorded while the voltage U was alternatively set to positive and negative values of increasing modulus (polarity refers to *cis*-side). For each section of positive voltage, the current has a significant non-zero value, but remains almost zero for negative voltage. This reveals the formation of pores, which are induced by electrical field with the proper *cis-trans* direction, and are removed upon field inversion. Fluctua-

tions of current have been already analyzed [6]. Here, we focus on the average value and compute for each voltage-section the average conductance $g = I/U$ as a function of T and U. Fig.1 shows a typical result. At this ionic strength the conductance of a single pore is typically 1 nS [6], so the dashed-line in Fig.1 might correspond to the peptide-poration transition. Note that its temperature dependence is the opposite of thermally activated processes such as electroporation occurring without peptide. In addition, the asymmetry regarding polarity can only be explained by the asymmetric peptide addition. This makes us confident that the dashed-line in Fig.1 coincides with the peptide-poration transition.

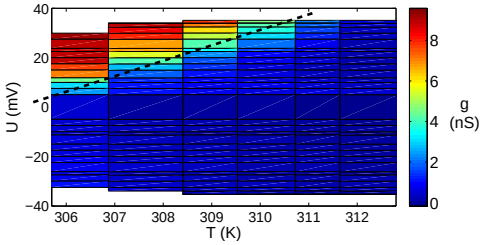

FIG. 1. Conductance g of DPPC/alamethicin vs. temperature T and electric potential U of the alamethicin compartment. The dashed-line has a slope equal to 6 mV/K.

The temperature dependence of the transition reveals the role of entropy, whereas the voltage variation shows an electric energy term. With the pore-free state as reference, the Gibbs free energy per pore is $G = H + QU - TS$, with H the enthalpy gain for one pore opening, S and QU the entropy loss and electrical work, respectively. At equilibrium $H + QU^* = T^*S$, leading to : $dU^*/dT^* = S/Q$. Here we found $S/Q \simeq 6\,\mathrm{mV/K}$, or:

$$TS/kT \simeq 70 \times Q/\mathrm{e} \qquad (1)$$

with k the Boltzmann constant and e the electron charge.

Due to its rodlike α-helix structure, alamethicin carries a global permanent electric dipole $\mu_\mathrm{P} = 15\,\mathrm{e\AA}$ [4], which could be viewed as responsible for voltage effects. This idea supposes a parallel (rather than anti-parallel) orientation of peptides forming a pore. If this were correct, assuming 6 peptides per pore [8] gives $Q = 6\mu_\mathrm{P}/z$ and Eq.1 would lead to $TS \simeq 125\,kT$. This is nonphysical and in turn pleads for of an antiparallel orientation of dipoles so that their moments cancel. This is not so amazing since : 1) although aligning one peptide dipole to the field would save $\sim 1\,\mathrm{kT}$, it would cost much more by confining parallel dipoles in repulsive interaction to form a pore; 2) the peptide dipole cannot explain the unequal sensitivity to polarity as it can favorably align with the field whatever its direction; 3) some peptides form voltage-facilitated pores despite them having no dipole [9]. So, the driving force for voltage-induced peptide-poration necessarily originates from the membrane.

Neutron reflectivity : Little is known about lipid bilayers under electrical field. Structural effects were observed by infrared spectroscopy [10] or neutron reflectivity [11] on dried stacked assemblies of bilayers. For high electric field ($\gtrsim 10^8\,\mathrm{V/m}$) results suggest the alignment with the field of phospholipid zwiterionic head-groups. Could similar effects occur at lower field (i.e. comparable to the natural transmembrane field) for fully hydrated head-groups ? Neutron reflectivity experiments have been reported on floating bilayers near a solid-water interface [12]. On such membranes, undulations that increase in amplitude with electrical field, dominate the reflectivity spectrum and likely hide more subtle changes.

To overcome this issue, we performed neutron reflectivity (EROS/LLB spectrometer) on single monolayers adsorbed at the interface between saline heavy water ([KCl]=1 M) and conductive silicon wafer allowing the electric field to be applied. The experimental setup is described in ref. [13]. Si-wafers were silanized following ref. [14] using octadecylthrichlorosilane (OTS). DPPC-monolayer was deposited on silanized wafer in the "liquid-expanded" (LE) phase by a modified Langmuir-Shaefer technique avoiding contact of the deposited layer with air. Deposits were done at 25°C at the controlled number density of $1/86\,\mathrm{\AA}^2$ i.e. comparable to that of aqueous bilayers and ensuring the fluidity of the film. The overall capacitance was measured as $C = 1\,\mu\mathrm{F}$. The electric field in the monolayer is $E = (C/A\epsilon_m)U$, with $\epsilon_m = 3\epsilon_0$ the membrane permittivity, ϵ_0 that of vacuum and $A = 12.6\,\mathrm{cm}^2$ the area. One gets : $E = 30 \times 10^6\,\mathrm{V/m}$ for $U = 1\,\mathrm{V}$ that is comparable to natural transmembrane field. Reflectivity measurements were done at $T = 23°\mathrm{C}$ for wafer potential $U = 0, -1, +1\,\mathrm{V}$. Their reliability was checked at least three times. We always observed the following behavior (Fig.2) : spectra divided by the Fresnel reflectivity show characteristic oscillations, which are fully superimposable for $U = 0$ and $-1\,\mathrm{V}$, but are unambiguously shifted to low values of transfer vector q for $+1\,\mathrm{V}$. This shift is irreversible at this temperature. Fitting in detail the reflectivity-data is out of the scope of this letter. Here, it is sufficient to note that the shift to low-q corresponds to thickening of the overall layer by :

$$\Delta z \simeq \left(1/q^*_{+1\mathrm{V}} - 1/q^*_{0\mathrm{V}}\right) \times 2\pi = (3.6 \pm 0.2)\,\mathrm{\AA} \qquad (2)$$

with q^* the positions of first maxima. This thickening is the opposite of what is expected for capacitive dielectric compression. The asymmetry regarding polarity necessarily involves the only permanent dipoles in the system, i.e. those of head-group zwitterions of moment $\mu_\mathrm{L} = 4\,\mathrm{e\AA}$ [15]. Given the size of head-groups [16], Eq.2 is fully consistent with their orientation almost parallel to the plane at 0 or -1 V and aligned to the field for +1 V. Due to incompressibility, thickening goes with area shrinking of the monolayer. Transposed to a bilayer this shrinking only concerns the leaflet on the side of negative potential and tends to bend the membrane with the con-

vexity on positive-side, i.e. the side of voltage-facilitated peptide-poration (Fig.1). In case of a held bilayer, the shrinking is not real but only rises the tension of the corresponding leaflet and the spontaneous curvature. In case of a laterally free monolayer, it is real and probably causes the transition from LE to a more condensed phase. This likely explains the observed irreversibility.

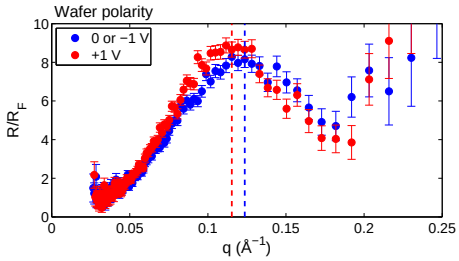

FIG. 2. Reflectivity of DPPC-monolayer at D_2O/OTS-Si-wafer interface divided by the Fresnel's reflectivity of D_2O/Si vs. transfer vector q. Dashed lines mark oscillations maxima.

Model for peptide-poration - 1) Spontaneous pores : Spontaneous opening of one pore in lipid bilayer involves a mechanical energy [3] :

$$E_m = \gamma_e 2\pi r - \sigma_{m0}\pi r^2 \tag{3}$$

with r the radius of the pore, γ_e its edge-energy and σ_m the bare-membrane tension. E_m reaches a maximum $E_m^* = \pi\gamma_e^2/\sigma_{m0}$ for $r_m^* = \gamma_e/\sigma_{m0}$ below which the pore tends to close and beyond which it grows indefinitely.

2) Tension-driven mechanism [2] : In this now classical model, when a peptide adsorbs onto the bilayer, it pushes away the lipid head-groups in order that its hydrophobic part meets the heart of the bilayer. Thus, a symmetric adsorption on both leaflets expands the area A of the bilayer. For small area number density x of peptides : $\Delta A/A = xA_P/2$, with A_P the area per peptide. The key point is that this is supposed to equally increase the membrane tension $\sigma_m = k_s(\Delta A/A)$, with k_s the stretching modulus (implicitly in ref.[2], this amounts to considering the membrane as a canonical ensemble) :

$$\partial\sigma_m/\partial x = k_s A_P/2 \tag{4}$$

σ_m would increase until E_m^* is small enough to allow thermal fluctuations to form pores larger than r_m^*. Let us denote x^* the corresponding x-value. Assuming that peptides incorporated into the membrane at the pore edge have no effect on surface tension, it can be shown that further increase of x enriches the incorporated-peptide population only (i.e. their area number density x_i increases whereas $x - x_i = x^*$ is constant), similarly to a phase transition. Thus, beyond x^* the contribution of adsorbed-peptides to E_m in Eq.3 is $E_{ap} = -\pi r^2(x^* k_s A_P/2)$. If the line density ρ of peptides on the pore rim is constant : $x_i = n_i\rho 2\pi r$, with n_i the area number density of pores, then E_{ap} splits into two terms :

$$E_{ap} = -\left(xk_s A_P/2\right)\pi r^2 + \left(2\pi^2 n_i \rho k_s A_P/2\right) r^3 \tag{5}$$

With Eq.3 in mind, the term in r^3 allows pores of radius larger than r_m^* to be stable. This model faces two problems : 1) these peptides are amphipathic, so their surface excess is positive (they populate the surface rather than the membrane bulk) and from the Gibbs adsorption isotherm $\partial\sigma_m/\partial x$ should be negative. Indeed, Eq.4 should be revised as the grand canonical ensemble is likely to be more adequate ; 2) Electric field applies a capacitive pressure on bilayer that lowers its effective tension [17]. So, how to account for voltage-induced pores ?

3) Spontaneaous curvature : Here, we propose to solve the above issues by noting that when peptides are asymmetrically adsorbed on one given bilayer leaflet, they tend to increase its "natural" area and thus the spontaneous bilayer curvature, c_0, with the same convexity orientation as *cis-trans* electric field does. The curvature energy stored by a flat bilayer is $E_{el} = A\frac{1}{2}k_c c_0^2$, with k_c the bending elastic modulus. For spherical curvature $c_0 \ll z^{-1}$, $c_0 = \Delta A/A2z$, with ΔA the area difference between leaflets. For x peptides per unit area adsorbed on one given leaflet, we obtain : $\Delta A/A = xA_P$. Thus $c_0 = x\mathcal{L}$ and $E_{el} = A\frac{1}{2}k_c\mathcal{L}^2x^2$, with $\mathcal{L} = A_P/2z$. On the contrary, peptides in pores equally contribute to both leaflets and do not affect E_{el} (as for σ_m within the tension-driven mechanism). So, with one pore involving $\rho 2\pi r$ peptides, the curvature energy is reduced to $E_{el} = A\frac{1}{2}k_c\mathcal{L}^2(x - \rho 2\pi r/A)^2$. The difference is :

$$E_{ap} = -k_c\mathcal{L}^2 x\rho \times 2\pi r, \tag{6}$$

instead of Eq.5. Note that by establishing bridges between the two bilayer leaflets, pores increase their coupling that in turn increases the bending rigidity. This effect has been observed by neutron spin echo [18]. It can be the origin of cooperativity of pore opening.

4) Pore edge : Although antimicrobial peptides are in many cases rodlike, they do not display the symmetry of a solid of revolution with respect to their amphiphilicity. Instead, the projection in the plane normal to their axis shows a bipolar repartition [19]. When peptides are parallel to the pore axis, their interfacial energy $\mathcal{E}_P$ is minimized when they are at the pore edge with their hydrophilic zone facing the channel. Consider the projection of the channel in the membrane plane as a polygon of angle α with peptides as vertices, we note α^* the angle of the same vertex that includes the hydrophilic zone of the peptide ($\alpha^* < \pi$ otherwise hydrophilicity dominates and peptides likely do not adsorb). $\mathcal{E}_P$ is minimum for $\alpha = \alpha^*$, occurring for the optimal pore radius $r^* = 1/\left(\rho(\pi - \alpha^*)\right)$. Deviation from α^* increases $\mathcal{E}_P = r_P z\Delta\alpha\Delta\gamma$, with r_P the radius of peptide-rod, $\Delta\alpha = \|\alpha - \alpha^*\|$, and $\Delta\gamma > 0$ the surface tension difference between heterophilic and homophilic contacts

($\Delta\gamma_{\alpha<\alpha^*} = \gamma_w^h - \gamma_w^w$ and $\Delta\gamma_{\alpha^*<\alpha} = \gamma_h^w - \gamma_h^h$; superscripts h and w tag for peptide hydrophobic h or "waterphilic" w zones; subscripts for the facing medium i.e. either hydrocarbonated lipid tails h or water w). For sake of simplification let us assume that $\gamma_h^h = \gamma_w^w = 0$ and $\gamma_w^h = \gamma_h^w = \gamma$. The interfacial energy per pore $E_{\rm int} = \rho 2\pi r \mathcal{E}_{\rm P}$ is thus :

$$E_{\rm int} = 2\pi r_{\rm P} z \times \gamma \times \|1 - (r/r^*)\| \qquad (7)$$

$E_{\rm int}$ adds to the line tension in Eq.3. Doing so, it increases both r_m^* and E_m^*, but also introduces a minimum for E_m at $r^* < r_m^*$, allowing the pore to be stable without needing to cross the energy barrier E_m^*.

5) Electrical work : The electrical field tends to orientate lipid head-groups of the "negative-side" leaflet. The corresponding potential electrical energy is exactly balanced by an asymmetric tension of this leaflet of energy $E_{\rm leaf} = (A/A_{\rm L})\mu_{\rm L} U\cos(\theta)/z$, with $A_{\rm L}$ the area per lipid and θ the angle of head-groups dipoles with the field. When a pore opens, this tension is relaxed by $E_{\rm leaf}\pi r^2/A$. This leads to a simple expression for the electrical work of lipid dipoles coming with one pore opening :

$$W_{\rm L} = -\left(\pi r^2 \mu_{\rm L}\cos(\theta)/A_{\rm L} z\right) U \qquad (8)$$

$W_{\rm L}$ amounts to removing all the lipid dipoles in the area πr^2. Its analogous to take off the charges accumulated at the surface of the dielectric, $-U^2\epsilon_m\pi r^2/2z$, is always negligible compared to $W_{\rm L}$ for usual voltages. Eq.8 is thus the dominant electrical contribution. Note that this could be also relevant for peptide-free electroporation.

6) Entropy : The translational entropy of $N_{\rm L}$ lipids in an area A is $kN_{\rm L}\ln(eA/N_{\rm L}A_L)$. For a held membrane under tension, the opening of a pore of area πr^2 only relaxes a bit the tension and lets the overall area unchanged. So, the accessible area is reduced by the "excluded-area" of the pore. The entropy cost is :

$$-TS_{\rm L} = kT\pi r^2/A_{\rm L} \qquad (9)$$

As for the translational entropy of adsorbed peptides, it is also reduced by this excluded-area but also by the immobilization of peptides in pores assumed immobile. The resulting cost per pore is : $-TS_{\rm P} = kTx\,(x/x^*)\,\pi r^2$. As $x/x^* \simeq 1$ and $x \ll 1/A_{\rm L}$, thus $S_{\rm P} \ll S_{\rm L}$. It can be checked that it will be the same for all contributions of peptides to the entropy loss (rotational or conformational freedom etc). This is due to lipids outnumbering peptides and to the extensiveness of entropy. Eq.9 is thus the dominant contribution to the entropy cost of one pore.

Finally, the sum of Eq.3 and Eq.6 to 9 estimates the main contributions in the free energy per pore. In particular, this model allows us to account for the voltage-temperature dependence of pore opening : from Eq.8 and 9, one expects at the transition $\partial U^*/\partial T^* = k/\left(\mu_{\rm L}\cos(\theta)/z\right)$. With $\theta = 77.4°$ [20], one obtains $\partial U^*/\partial T^* = 5\,{\rm mV/K}$, in very good agreement with the result in Fig.1. Here, one understands that beyond an epiphenomenon, voltage effects amount to put the pore-opening transition in the correct temperature-window.

Voltage-induced peptide pores always open with *cis*-to-*trans* electric field regarding peptide. In this letter, consistently with our experiments, we propose that this asymmetry, as well as the electric work, originate from the membrane rather than from the peptide. We also argue that the main contribution to the entropy cost comes an "excluded-area" effect on lipid entropy. Thus, our results place the membrane behavior in central position for the energetics of pore opening and thus could have a more general impact in modeling large molecule incorporation.

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